Development of Selective Antisera for Muscarinic Cholinergic Receptor Subtypes

BARRY B. WOLFE AND ROBERT P. YASUDA

Department of Pharmacology
Georgetown University
School of Medicine
3900 Reservoir Road N.W.
Washington, D.C. 20007

Muscarinic cholinergic receptors belong to the family of G-protein–coupled receptors that modulate several second messenger systems including those of cyclic AMP and inositol trisphosphate. The actions of drugs on these receptors were initially described by Dale,[1] and subsequently large numbers of compounds have been shown to be agonists or antagonists at these receptors. In the 1970s and 1980s several investigators suggested on the basis of pharmacological data that subtypes of muscarinic receptors must exist; Goyal and Rattan[2] coined the terms M1 and M2 muscarinic receptors to refer to receptors having high and low affinity, respectively, for the drug pirenzepine. Later workers further subdivided the M2 class of receptors on the basis of selective affinity for the compound AF-DX-116 and defined the M3 class of receptors having low affinity for both AF-DX-116 and pirenzepine.[3]

During the late 1980s several laboratories reported cloning the cDNA and/or genes encoding muscarinic receptors.[4–10] In all, five distinct genes were identified and these molecularly defined subtypes were given the nomenclature m1–m5, using lowercase m to distinguish them from the pharmacologically defined M1–M3 receptors described above. The m1, and to some extent the m4, receptor corresponded with the M1 receptor; the m2, and to some extent the m4, receptor corresponded with the M2 receptor; the m3 receptor corresponded with the M3 receptor; it was unclear where the m5 receptor would be classified pharmacologically. Thus, the pharmacological classification methodology available does not have the tools to define unequivocally a given molecularly defined subtype of muscarinic receptor. In response to this problem, we embarked on a program to develop a set of antisera that selectively recognizes each of the five molecularly defined subtypes.

The general approach to obtaining selective antisera was to determine which parts of the primary sequence of the proteins, as deduced from the DNA sequence, were unique to each subtype and to utilize proteins or peptides based on these sequences as antigens. The muscarinic receptors, as for all known G-protein–coupled receptors, are monomers that have seven membrane-spanning regions with the N-terminus on the outside and the C-terminus on the inside of the cell. In general, the regions that are most unique between receptor subtypes include the N-terminal region, the third intracellular loop (I3), and the C-terminal region. Among these the I3 loop is large (approximately 170–230 amino acids) and, with the exception of the first 10 to 15 amino acids nearest the membrane, is quite different for all five subtypes. In addition, the I3 loop is fairly hydrophilic, a property that often increases the antigenicity of a protein. This region was thus chosen as a good immunogen to raise antisera against.

The cDNA encoding the I3 loops of each of the five muscarinic receptor subtypes was subcloned into expression vectors. Different vectors were used for the proteins because the experiments were performed over a two-year period in which new and better vectors became available. Thus, the m1, m2, and m4 cDNA was cloned into pRIT, an expression vector that is driven by a strong, constitutively-active promoter and expresses the IgG-binding domains of protein A.[11-13] Thus, the resultant fusion proteins were easily purified using an IgG-Sepharose column. For the m5 receptor the cDNA was cloned into pET3, which has an inducible promoter that drives expression levels to extremely high levels.[13] The protein produced from this vector does not, however, have an "affinity handle," and it was thus purified by size-exclusion chromatography. The m3 receptor cDNA initially proved difficult in that the proteins expressed using pRIT and pET3 were both badly degraded. Thus, initial antisera were raised to a unique small peptide corresponding to a region in the C-terminus.[14] In later experiments, however, the cDNA encoding the I3 loop of the m3 receptor was cloned into the vector pGEX3, which has an inducible promoter, and the resultant protein is fused to the enzyme glutathione-S-transferase (Li and Wolfe, unpublished data). The fusion protein is thus easily purified over a glutathione-agarose column.

Using these fusion proteins, or peptide in the case of m3, we injected rabbits; antisera were tested to determine whether antibodies were present that selectively recognized a specific subtype of muscarinic receptor. The assay that proved to be the most fruitful was immunoprecipitation of the radiolabeled receptor. As shown in FIG. 1, in this assay, receptors in membrane preparations are first labeled at 32 °C with the high-affinity muscarinic antagonist [^{3}H]QNB. This ligand is very specific for muscarinic receptors in that it does not seem to bind to other sites and has very low levels of "nonspecific" binding.[15] This ligand seems to have similar affinities for all five subtypes of muscarinic receptor with a K_d value near 10 pM (Wolfe, unpublished data). Receptors are labeled with a concentration (0.5–2 nM) of [^{3}H]QNB that occupies nearly all receptors. Samples are then cooled to 4 °C at which temperature the ligand has negligible dissociation over a two-day period.[11] The labeled receptors are then solubilized in digitonin and a high speed supernatant is produced. This supernatant containing the labeled receptors is incubated with the putative antiserum for 40–55 h at 4 °C. The IgG molecules in the antiserum are then complexed to Protein A on the surface of fixed bacteria to allow for the protein A/IgG/receptor/[^{3}H]QNB complex to be easily sedimented in a microcentrifuge.[11]

For these immunoprecipitation experiments, cells transfected with the cDNA encoding a single, defined subtype were used as a source of muscarinic receptors. It was found that a given antiserum would immunoprecipitate approximately 90% of the labeled receptors from cells expressing the appropriate receptor and less than 2% of labeled receptors from cells expressing the inappropriate receptor.[11-14] Thus, the antisera were selective and quantitative in their ability to recognize each subtype of muscarinic receptor and represented tools with which to study the distribution and density of the muscarinic receptor subtypes.

A number of studies have been carried out using these antisera. Initial experiments determined the general distribution of muscarinic receptor subtypes in rat brain and certain peripheral organs. Thus, as shown in FIGURE 2, all subtypes except m5 are expressed at reasonably high (> 200 fmol/mg protein) levels compared to a number of other receptors in the rat brain. The m1 receptor, for example, is expressed at high levels (> 800 fmol/mg) in the rostral portions of the rat brain including the cortex, hippocampus, striatum, and olfactory tubercule. In the mid-brain, and pons-medulla it was expressed at much lower levels, and in cerebellum m1 receptors are only barely detectable. The profile for the m2 receptor, on the other

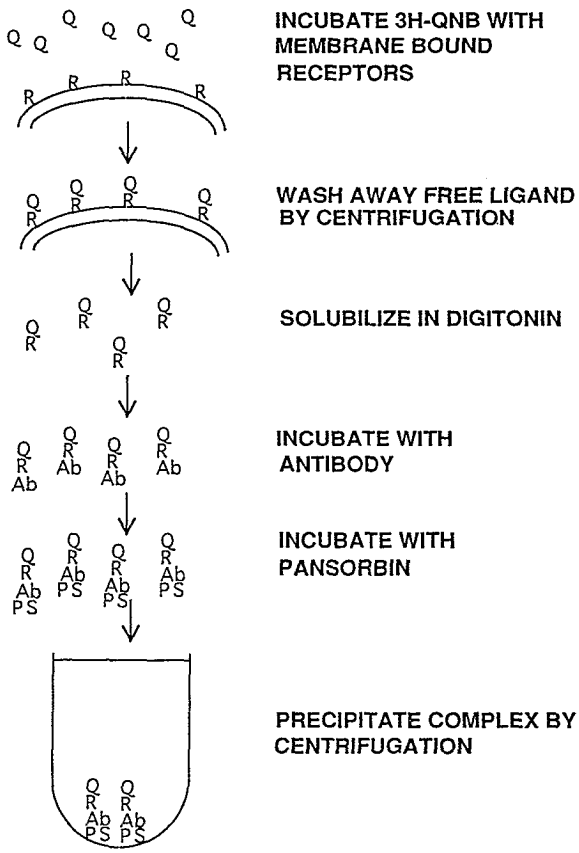

FIGURE 1. Method for immunoprecipitation of radiolabeled muscarinic receptor subtypes. Membrane-bound muscarinic receptors (R) are incubated at 32 °C with [^{3}H]QNB (Q) at a concentration (0.5–2 nM) that will occupy essentially all of each subtype. Samples are cooled to 4 °C, centrifuged, and the membranes washed to remove free ligand. Labeled receptors are solubilized in 1% digitonin and the high speed (80,000 × *g*) supernatant is incubated with a subtype-selective antiserum (Ab). Following 40 to 55 h of incubation at 4 °C, Pansorbin (PS; fixed bacteria expressing protein A on their cell wall) is added, and the [^{3}H]QNB/receptor/antibody/Pansorbin complex is sedimented to separate labeled subtypes.

hand, shows that this receptor subtype is distributed throughout the brain in a relatively homogeneous manner with levels in most areas being around 250–400 pmol/mg. In the cerebellum m2 receptor density is only about 150 pmol/mg, but this still represents the majority of the muscarinic receptors expressed in this brain area. The m3 subtype is expressed in a pattern somewhat similar to that seen for m1 albeit at lower levels. Thus, there is a clear rostral-to-caudal gradient of m3 receptor expression. The m4 receptor is most highly expressed in the striatum where levels are nearly 1300 fmol/mg, the highest level of any single subtype in any area of the brain

examined. This subtype is also poorly expressed in the caudal portions of the brain. The m5 muscarinic receptor was found, on the other hand, to be in very low abundance anywhere in the brain with reliably detectable values (15–25 fmol/mg) found only in the hippocampus, striatum, and midbrain.

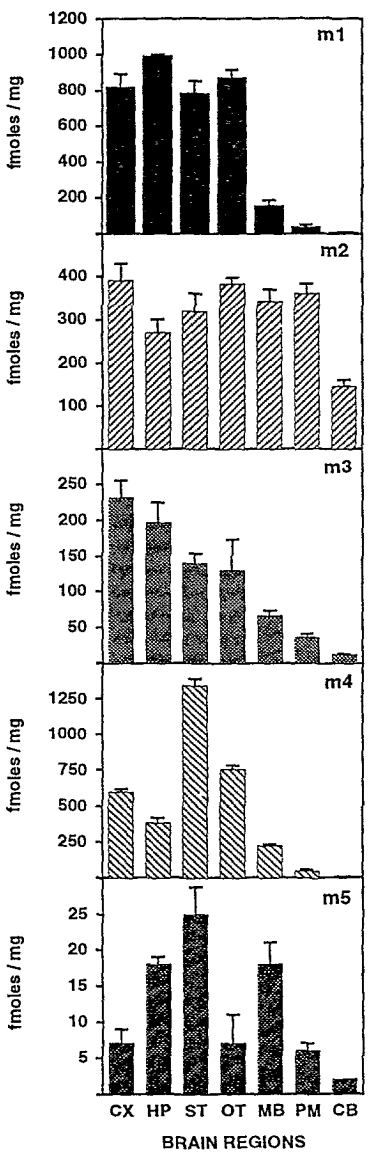

FIGURE 2. Regional distribution of muscarinic receptor subtypes in rat brain. Rat brain was dissected into seven regions: cortex (CX), hippocampus (HP), striatum (ST), olfactory tubercule (OT), midbrain (MB), pons-medulla (PM), and cerebellum (CB). Midbrain contained structures such as the thalamus, hypothalamus, and *substantia nigra*. Tissues were processed as described in FIGURE 1 to obtain the percent of each subtype in a given tissue. Total density of muscarinic receptors was determined by [^{3}H]QNB binding. The percentage of a specific receptor subtype in a given tissue was multiplied by the total density of muscarinic receptors in that tissue to obtain the density (in fmol/mg protein) of the subtype. Data are adapted from Wall *et al.*,[11,14] Li *et al.*,[12] and Yasuda *et al.*[13]

It is interesting to compare the distribution of the protein determined by the immunoprecipitation studies to the distribution of the mRNA described using *in situ* hybridization techniques. Thus, the m1 mRNA distribution is not dissimilar to that found for protein distribution.[8,11] The distribution of m2 mRNA and protein are, however, quite different with the mRNA being concentrated in areas of cholinergic cell bodies whereas the protein is found to be widely distributed.[8,12] Additionally, the levels of m2 mRNA have been found to be very low relative to m1, m3, and m4.[8] Interestingly, the levels of m2 protein are higher than those of the m3 protein in many areas of the brain including, for example, the hippocampus in which the mRNA levels are quite the opposite.[8,12,14] Therefore, mRNA distribution and density do not necessarily predict protein distribution and density. On the other hand, the mRNA for m4 receptors has been shown to be very highly expressed in the striatum,[8] an area found to be very rich in m4 protein. Similarly, m5 mRNA was initially reported not to be found in rat brain but a subsequent study demonstrated the expression of this mRNA in the substantia nigra, pars compacta,[16] localized, presumably, in the dopaminergic neurons projecting to the striatum. Immunoprecipitation studies found the m5 receptor at low levels in the midbrain (which would include the substantia nigra) and the striatum, suggesting a predictive relationship between mRNA and protein for the m5 receptor.

Other studies utilizing the selective antisera have focused on the regulation of receptor subtypes in rat brain following loss of cholinergic input. A guiding hypothesis for these studies is that if neurotransmitter access to a receptor is blocked, either by blocking the receptor or destroying the neurons containing the neurotransmitter, the receptor will often respond by up-regulating its density. If this hypothesis is correct and uniform for all receptors (probably a stretch), then the level of up-regulation gives some estimate of the level of neurotransmitter "tone" in the normal situation. On the other hand, if a receptor is expressed on a neuron that is destroyed by a lesion, one would expect the density of that receptor to decrease. Two paradigms have been examined. The first involves chronic administration of the muscarinic receptor antagonist atropine.[17] This drug is nonselective and binds with high affinity and specificity to all five subtypes of muscarinic receptor. When rats were treated for 14 days with high levels of atropine and the effects on receptor densities were examined using immunoprecipitation, it was found that only some subtypes of muscarinic receptors were affected (FIG. 3). Levels of m2 receptors were unaffected whereas levels of m1 and m4 receptors increased modestly by about 10 to 20%. On the other hand, levels of m3 receptors were up-regulated by nearly 70%, indicating a high degree of "plasticity" and a high degree of tone for this receptor subtype. In a second, related paradigm, cholinergic tone was stopped by lesioning the cholinergic neurons innervating the dorsal hippocampus.[18] Either 10 or 24 days after the cholinergic lesion the densities of muscarinic receptor subtypes were determined. At both times the results were identical and data were combined (FIG. 3). In general, results were similar to those obtained by chronic blockade with atropine. Thus, m1 and m4 receptor densities were increased modestly by 10 to 30% whereas m3 receptor density was more strongly up-regulated by 77 percent. On the other hand, levels of m2 receptor *decreased* by more than 20% in contrast to the observed result from chronic atropine blockade. These data could be interpreted to indicate that, as concluded for the atropine experiment, inasmuch as the m1 and m4 receptors are not as "plastic" as m3 receptors they may not receive as strong a tone as the m3 receptors under normal conditions. The decrease in m2 receptors can be interpreted to indicate that some m2 receptors reside on the cholinergic nerve terminals that were destroyed by the lesion. This is consistent with much of the data demonstrating a

functional presynaptic autoreceptor on these terminals that regulates acetylcholine release, which may be an m2 receptor.[19]

Lastly, experiments have been performed examining the normal developmental profile of the subtypes of muscarinic receptor in whole rat brain.[20] In general, each of the five subtypes developed along approximately the same time course with levels being very low at 3 to 4 days after birth, but rising rapidly over the next several days to reach adult levels by about 14 to 20 days after birth. This developmental profile is

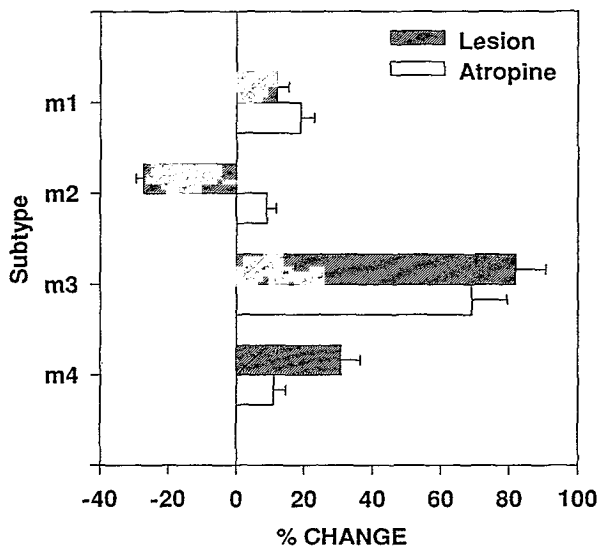

FIGURE 3. Changes in receptor subtype densities following either chronic blockade with atropine or chronic denervation by fimbria-fornix lesion. In one set of experiments rats were unilaterally lesioned by cutting the fimbria-fornix and the rostral supracallosal stria/cingulum bundle, pathways by which cholinergic neurons innervate the dorsal hippocampus. Either 10 or 24 days post lesion, dorsal hippocampus tissue was taken, and the densities of muscarinic receptor subtypes were determined as described in FIGURE 1. Data shown in the hatched bars (adapted from Wall *et al.*[18]) are presented as percent change from the control (unlesioned) side. In other experiments, rats were treated with atropine to block all five subtypes of muscarinic receptors for 14 days. Cortex and dorsal hippocampus were assayed for subtypes of muscarinic receptors as described above. Data shown in open bars (adapted from Wall *et al.*[17]) are presented as percent change from the control (sham-treated) rats. All values were statistically ($p < 0.05$) different from control except m2 receptors in atropine-treated tissues.

similar to that of carbachol-stimulated phosphoinositide hydrolysis,[21] suggesting that the appearance of receptors is responsible for the appearance of responsiveness. On the other hand, Lee *et al.*[21] reported that carbachol-mediated inhibition of adenylyl cyclase does not appear before age 14 days. Thus, for this signal transduction system and the receptors (m2 and m4) that mediate it, the appearance of receptors does not mandate the appearance of functional response and that the latter is more likely tied to the regulation of receptor/G-protein coupling.[21]

In conclusion, the development of a set of antisera that can localize and quantify each of the five subtypes of muscarinic cholinergic receptor has led to a number of novel observations. Future experiments examining, for example, the relationship of these receptors and the loss of cognitive abilities in aged rats or the decline in cognition in humans with Alzheimer's disease may provide new data with which to formulate hypotheses regarding therapeutic intervention in various disease states.

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